Journal of Experimental Botany, Vol. 58, No. 12, pp. 3395–3406, 2007
doi:10.1093/jxb/erm188 Advance Access publication 4 October, 2007
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Gene expression profiles for cell wall-modifying proteins associated with soybean cyst nematode infection, petiole abscission, root tips, flowers, apical buds, and leaves

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Received 5 March 2007; Revised 22 May 2007; Accepted 11 June 2007

Abstract

Changes in transcript accumulation for cell wall-modifying proteins were examined in excised soybean root pieces colonized by soybean cyst nematodes (SCN), *Heterodera glycines*, using RT-PCR and soybean Affymetrix GeneChips. Sequence-specific PCR primer pairs were prepared from sequence data for core sequences in the GenBank soybean database and consensus sequences derived from the assembly of soybean ESTs. In addition, to identify previously uncharacterized soybean transcripts, degenerate primers were prepared for conserved motifs in cellulases (endo-1,4-β-glucanases, EGases) and polygalacturonases (PGs) and these were used to amplify segments of transcripts that were then extended with 3′ and 5′ RACE. Several novel EGase and PG transcripts were identified. Gene expression patterns were determined by real-time RT-PCR for 11 EGases, three expansins (EXPs), 14 PGs, two pectate lyases (PLs), and two xyloglucan endotransglucosylase/hydrolases (XTHs) in soybean roots inoculated with SCN, non-inoculated roots, serial dissections of root tips, leaf abscission zones, flowers, apical buds, and expanding leaves. A large number of genes associated with cell wall modifications are strongly up-regulated in root pieces colonized by SCN. However, in contrast to most of the transcripts for cell wall proteins, two XTH transcripts were specifically down-regulated in the colonized root pieces. Gene expression in serial dissections of root tips (0–2 mm, and 2–7 mm) and whole roots indicate that the SCN up-regulated genes are associated with a wide range of developmental processes in roots. Also of interest, many of the cDNAs examined were up-regulated in petiole abscission zones induced to abscise with ethylene.

Key words: Cell wall, cellulase, cyst nematode, expansin, *Glycine max*, *Heterodera glycines*, pectate lyase, polygalacturonase, soybean, XET.

Introduction

The interactions between soybean cyst nematode (SCN), *Heterodera glycines*, and its host are biologically interesting and of great commercial importance because SCN is currently the most economically damaging pest of soybean (Wrather and Koenning, 2006). SCN is an obligate parasite of soybean roots and feeds only after it has penetrated the root, migrated to the vascular bundle, and induced the formation of a complex multinucleated feeding structure, syncytium (Jung and Wyss, 1999). SCN possesses a protrusible hollow stylet that is capable of physically disrupting root cells to aid in its migration through the root. The stylet is also the conduit for secretions from the nematode oesophageal glands (Davis *et al*., 2004). Secretions were promptly recognized as significant for further study because of their potential role as virulence factors that could alter host cell structure and biology (Hussey, 1989). Some of the first oesophageal secretions identified were cellulases (endo-1,4-β-glucanases, EGases) (Smant *et al*., 1998; de Boer *et al*., 1999). These nematode EGases are secreted from the subventral oesophageal gland of the juvenile nematode as it migrates towards the vascular bundle (Wang *et al*., 1999).

The cyst nematode feeding structure forms by the dissolution of plant cell walls and membranes between

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adjacent cells to form one large multinucleated cell with a thickened enclosing cell wall (Jung and Wyss, 1999). Extending inward from the cell wall are fingerlike ingrowths of the plasma membrane that presumably increase the surface area for nutrient uptake (Golinowski *et al*., 1996). The formation of the syncytium can incorporate as many as 200 plant cells into one long feeding cell that extends longitudinally along the vascular bundle (Jung and Wyss, 1999). It was hypothesized that the nematode might secrete the proteins needed to degrade the cell walls separating the cells that were incorporated into the syncytium. In addition to the aforementioned EGases, Gao *et al*. (2003) identified a pectate lyase (PL) in their oesophageal gland cDNA library. However, transcript abundance for the nematode EGases and PL declined with the initial appearance of the syncytium (de Boer *et al*., 1999; Gao *et al*., 2003). Their expression patterns fit better with cell wall degradation during nematode migration rather than syncytium formation. Moreover, antiserum prepared against an EGase from the potato cyst nematode, *Globodera rostochiensis*, detected antigen in the migratory path of the tobacco cyst nematode (TCN), *Globodera tabacum* spp *solanacearum* but not in the developing syncytia (Goellner *et al*., 2001). An alternative hypothesis was that the nematode displayed or secreted a signal that stimulated the host to produce the cell wall-degrading proteins used to form the syncytium. More recently, root knot and cyst nematode secretions have been characterized that have the potential to change markedly host gene expression at the nematode feeding site (Doyle and Lambert, 2003; Wang *et al*., 2005; Huang *et al*., 2006). One example is *Hg-SYV46*, an SCN gene, with similarity to *CLAVATA3* (*CLV3*) that participates in apical meristem differentiation in *Arabidopsis* (Olsen and Skriver, 2003; Fiers *et al*., 2005). Expression of the SCN SYV46 protein in *Arabidopsis* produces a wus-like phenotype much like overexpression of the *CLV3* gene in *Arabidopsis*. Another example is a 13 amino acid peptide (16D10) that is secreted from root knot nematodes (RKN) and stimulates growth when expressed in plant tissues (Huang *et al*., 2006). Evidence indicates that the16D10 peptide interacts with plant SCARECROW-like transcription factors. Thus, these and other nematode secreted proteins may directly or indirectly alter gene expression at the nematode feeding site.

Goellner *et al*. (2001) identified five tobacco EGases that were up-regulated upon infection with either TCN or RKN, *Meloidogyne incognita*. They furthermore demonstrated by *in situ* hybridization that three of the mRNAs accumulated to a high concentration in the RKN-induced feeding structure, giant cells, and two accumulated in the TCN-induced syncytium. In addition to accumulation in giant cells and syncytium, the same mRNAs were more concentrated in root tips, and lateral root primordia (Goellner *et al*., 2001). Moreover, in a genome-wide survey of transcript accumulation in *Arabidopsis* roots infected with RKN, an expansin (EXP) and a PL were noted as being two of three genes up-regulated by more than 15-fold in infected root tissue (Jammes *et al*., 2005). Recently, Wieczorek *et al*. (2006) examined the expression patterns for 29 different EXP genes in *Arabidopsis* roots infected with the cyst nematode *Heterodera schachtii*. They demonstrated by RT-PCR that at least 10 different EXPs were present in syncytial RNA at 5–7 days post inoculation (dpi) and GeneChip analysis indicated that at 15 dpi seven of these were significantly up-regulated when compared with non-inoculated control roots. These results substantiate the importance of host gene expression and cell wall modifications at the nematode infection site and in the development of the nematode feeding structure.

Cell wall modifications are an integral part of most, if not all, plant developmental and growth responses. Some of the most notable are fruit ripening, cell elongation, vascular differentiation, abscission, dehiscence, and aerenchyma formation. The gene families for cell wall-modifying proteins can be quite large. For example, Imoto *et al*. (2005) estimated that, in *Arabidopsis*, there are at least 67 genes encoding polygalacturonases (PGs), 26 PLs, 111 pectin esterases, 33 xyloglucan endotransglucosylase/hydrolases (XTHs), 24 EGases and other hydrolases, and 23 EXPs. Although the total number of genes reported for each gene family may differ, for example, 23 versus the 29 EXPs reported by Wieczorek *et al*. (2006), the large number of genes highlights the importance of cell wall modifications to plant growth and development (Imoto *et al*., 2005).

Here, the gene expression patterns for 11 EGases, three EXPs, 14 PGs, two PLs, and two XTHs are described. In addition to the basic goal of identifying genes that are up or down-regulated in SCN-colonized roots, the gene expression results for these plant cell wall-modifying proteins have been extended to include gene expression profiles in serial dissections of root tips, apical meristems, leaves, flowers, and petiole abscission zones. Abscission (organ separation) is a cell separation process requiring extensive degradation of the cell wall that may be of interest in comparison to cell wall breakdown that occurs during SCN colonization of soybean roots and formation of the syncytium. Several of the root-expressed genes were also expressed in the petiole abscission zones.

Materials and methods

Plant material, nematode inoculation, and RNA extraction

The SCN population NL1-RHg, (HG-type 7, race 3) has been maintained on greenhouse-grown soybeans at USDA, ARS, Beltsville, Maryland and can be obtained from the SCN Stock Center (T Niblack, University of Illinois, Champaign-Urbana, IL). The nematode inoculum was prepared as previously described by

Matthews *et al*. (2003). Seeds for *Glycine max* cv. Williams were germinated in Perlite (Geiger, Harleysville, PA) in the greenhouse and, after 2 weeks, seedlings were washed free of Perlite, combined into groups of six seedlings, and inoculated by pipetting 5000 J2/seedling as previously described (Puthoff *et al*., 2007). For the experiments where root pieces were excised, the inoculated roots were placed under a stereomicroscope to dissect out small root pieces displaying swollen female nematodes protruding from the root (Puthoff *et al*., 2007). The lateral roots and protruding root tips were trimmed away while still being observed under the stereomicroscope and the trimmed root pieces were then frozen in liquid nitrogen. Root pieces from non-inoculated roots incubated for the same interval of time were collected from similar positions in the root as the SCN-colonized root pieces, and then trimmed and frozen. The collection of intact whole roots from inoculated and non-inoculated roots was completed three times (three biological replications) and the collection of root pieces was performed twice (two biological replications). A separate batch of 18-d-old greenhouse-grown plants were used to collect whole roots and root tips serially dissected into sections of 0–2 mm and 2–7 mm proximal to the root apex. All root samples were stored at –70 °C. Frozen root samples were ground with a mortar and pestle cooled with liquid nitrogen. Powdered frozen samples were rapidly suspended in RNA extraction buffer using a Kinematica Polytron PT 1200 (Brinkmann Instruments, Westbury, NY). RNA from whole roots was extracted as previously described (Koehler *et al*., 1996). RNA from root pieces, root tips, and the whole roots associated with the root tip dissection was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA).

In the abscission experiments, 14-d-old explants were prepared by cutting the stems above the soil and then removing the cotyledons, leaf blades, and apical buds. The explants were placed in a beaker of water in a flow-through gas chamber with 25 μl l^{-1} ethylene in air at 25 °C. Abscission zones (2 mm) were collected from the base of the petioles after 24 h and 48 h of ethylene treatment and frozen in liquid nitrogen. Apical buds (approximately 2 mm) were collected from 14-d-old soybean plants and frozen in liquid nitrogen. Flowers at all stages of development and young expanding leaves (3–5 cm across) were collected from 2-month-old plants. RNA was extracted as described for root pieces using a Qiagen RNeasy Plant Mini Kit.

Cloning, sequence analysis, and preparation of primer pairs

First-strand cDNA was synthesized using Superscript III-First Strand Synthesis System (Invitrogen, Carlsbad, CA). PCR with degenerate primers was completed using annealing temperatures of 50 °C and 55 °C. The PCR products were separated in a low melting temperature agarose gel and the appropriate size fraction cut from the gel and cloned into the pGEM-T Easy vector (Promega, Madison, WI) (Sambrook *et al*., 1989). Clones were sequenced using the ABI Big Dye Terminator Cycle Sequencing Kit and run on the ABI Prism 3100 Genetic Analyser (Perkin-Elmer, Applied Biosystems, Foster City, CA). A GeneRacer Kit (Invitrogen) was used for 3′ and 5′ RACE to obtain sequences upstream and downstream from the PCR product. The sequences for some of the RACE reactions were fully confirmed and deposited in the GenBank database: Cel2 (DQ340397, DQ340402), Cel7 (DQ340398, DQ340399, DQ340400), Cel8 (DQ357226, DQ357227, DQ357228), Cel9 (DQ382353, DQ382360), Cel12 (DQ382354, DQ382355), PG6 (DQ382356, DQ382357), PG7 (DQ360494, DQ360495, DQ360496), PG11 (DQ340401), PL2 (EF432111, EF432112), and XET1 (DQ382358, DQ382359). In several of the RACE reactions, two amplification products were obtained that were in the expected size range. In this case, both fragments were collected and cloned. After complete sequence confirmation, the two different size PCR fragments in the same PCR reaction were different lengths and often included a few mismatches when the sequences were aligned. Different polyadenylation sites for the same gene could explain different length PCR products, but the mismatches suggest that they represented two separate genes (paralogues). Multiple copies of genes are common in soybean, a tetraploid, and several cDNAs that are highly similar in the 5′ UTR and ORF but differ in the 3′ UTR have been discovered. However, in all cases except PG11, the 5′ and 3′ RACE sequences were submitted to the GenBank database as separate sequences because, although the overlap of the sequences might be an identical match over 100 or more nucleotides, it was not possible to be certain that the two clones were from the same gene or from highly similar paralogues.

Sequences were processed, aligned, and analysed using the GCG Wisconsin Package (Accelrys, San Diego, CA). In all cases except the scan for a signal peptide in Cel2, the default settings were used. In order to identify a putative signal peptide for Cel2 (accession number DQ340397), the minimum acceptance score for SPScan was lowered to 3.0. The software used to create the heat-map was described by Chiang *et al*. (2001) and is available at http://rana.lbl.gov/EisenSoftware.htm.

RT-PCR

PCR primer pairs were designed for multiple EGases, EXPs, PGs, PLs, and XTHs expressed in soybean roots. Primer pair specificity was initially tested by running the PCR products on an agarose gel to ensure that a single band of the expected size was obtained. The primer pairs were then used in real-time RT-PCR experiments to assure that a single dissociation peak predominated the melting temperature spectra after 35 and 40 cycles. If multiple dissociation peaks were observed, new primers were prepared and tested until a single peak predominated the spectra. The primer pairs were then tested in RT-PCR with RNA from nematode eggs and J2 (juveniles at stage 2) to ensure that none of the primers amplified an orthologous sequence in the nematode (data not shown). Primer annealing, extension, and denaturing temperatures of 60 °C, 70 °C, and 95 °C, respectively, were used for both standard PCR and real-time PCR.

For standard and real-time RT-PCR, the cDNA synthesis was completed with 5 μg of RNA using the Superscript III-First Strand Synthesis System for RT-PCR (Invitrogen). A single bulk cDNA synthesis reaction was used to reduce differences that might occur in the cDNA synthesis reaction and the cDNA diluted to a volume that could accommodate the real-time PCR for large sets of primer pairs. Real-time PCR reactions were completed using a Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) in an Mx3000P instrument (Stratagene). Forty cycles of real-time PCR were completed for all of the primer pairs and a 0.1 threshold for SYBR Green fluorescence normalized to the ROX reference dye was used to mark the number of PCR cycles needed to incorporate enough fluorescent compound into double-stranded DNA to cross this threshold (CT). The soybean elongation factor (GmEF1b), which is a part of the ribosomal protein translation complex, was considered to be a constitutively expressed mRNA and used as an internal control in the RNA samples. A series of dilutions for each cDNA sample was prepared and run with the GmEF1b primer pair to determine the efficiency of amplification for the GmEF1b cDNA in each of the cDNA synthesis samples. The log concentration (dilution factor) was plotted against the CT values and a regression line calculated for the dilution series for each RNA sample. Parallel regression lines for each RNA sample indicated relatively equal efficiency of amplification between RNA samples. It has been our experience and others (Wong and Medrano, 2005) that if the same batch of real-time PCR master mix is used for all amplifications, the efficiency of amplification of an internal control (e.g. GmEF1b) is

fairly constant, i.e. parallel regression lines. However, if different master mixes are used, the efficiency of amplification tended to be slightly different. To reduce this variation, master mixes were combined so that there was enough of a single batch of master mix to complete RT-PCR for the full series of RNA samples with several different primer pairs.

Although the regression lines for the GmEF1b dilution series were reasonably parallel, the regression lines for each RNA sample were offset from each other indicating that the concentration of the GmEF1b transcript in the RNA samples was slightly different for each sample. The CT values for each of the RNA samples were normalized to a value determined by comparing the GmEF1b regression line of the individual cDNA samples to the collective regression line of the combined RNA samples. Normalization corrects for inaccuracies in RNA quantification or cDNA synthesis between the various RNA samples. While a choice was made to perform this normalization, the resulting corrections tended to have a minimal effect on the expression patterns obtained.

Probe synthesis and GeneChip hybridization

Total RNA was isolated as described above. Probe synthesis, hybridizations, and analysis of the microarray images were performed at the GeneChip Facility at Iowa State University, Ames, IA as previously described (Puthoff *et al*., 2007).

Microscopy

The 50 μm thick longitudinal section was cut from a fresh non-fixed root tip. A 2 cm lateral root tip was placed in a slit cut into a piece of Styrofoam and then sliced longitudinally with a Lancer Vibratome 1000 (Lancer, St Louis, MO). The 0.5–1.0 μm sections were obtained from root tips fixed by immersion in 3% glutaraldehyde, 0.05 M sodium cacodylate buffer, pH 7.0, for 2 h at room temperature followed with 4 °C overnight. The roots were then rinsed in 0.05 M sodium cacodylate buffer six times over 1 h followed with post-fixation in 2% buffered osmium tetroxide for 2 h, dehydrated in a series of 20%, 40%, 60%, 80%, 95%, and 100% ethanol, and infiltrated with Spurrs low-viscosity embedding resin (Electron Microscopy Science, Fort Washington, PA). The root tissue was sectioned to a thickness of 0.5–1.0 μm on a Reichert/AO Ultracut microtome (Leica, Deerfield, IL) with a Diatome diamond knife and then stuck onto glass slides. Sections were then stained with 1.0% toluidine blue O in 1% sodium borate, rinsed briefly with water, air-dried, and then the embedding resin destained by soaking in 100% ethanol for 15 min.

Results

Identification of transcripts for cell wall-modifying proteins

Sequence-specific PCR primers were prepared for several consensus sequences generated by alignment of core nucleotide sequences and ESTs for cell wall proteins in the GenBank soybean database. PCR amplification was then used to identify primer pairs for transcripts expressed in SCN-colonized roots. To discover additional genes that were not already in the soybean database, degenerate primers specific for conserved regions in the alignments of transcripts for EGases and PGs from multiple plant species were prepared (see Supplementary Table S1 at *JXB* online). The degenerate primers were then used to RT-PCR amplify a mixed population of sequence fragments in pooled RNA extracted from SCN inoculated soybean roots at 2, 4, 8, 12, and 20 dpi. Clones related to 57 EGases and 57 PGs were sequenced and compared with each other to identify a set of unique sequences. Internal primers were prepared to each of the unique sequences and used in 3′ and 5′ RACE reactions to obtain clones for upstream and downstream sequences. In addition, Affymetrix GeneChip hybridizations indicated that several EXPs and PLs were strongly up-regulated in SCN-colonized root pieces (Puthoff *et al*., 2007). Three EXPs and two PLs were selected for further characterization. In total, gene-specific PCR primers were prepared for 11 EGases, three EXPs, 14 PGs, two PLs, and two XTHs (see Supplementary Table S1 at *JXB* online). All of the sequences used to design PCR primers, including novel sequences and consensus sequences for EST alignments, are included in a FASTA file (see Supplementary Table S2 at *JXB* online).

All of the N-terminal peptide sequences were searched for signal peptides for synthesis on the endoplasmic reticulum and possible secretion into the cell wall (von Heijne, 1983). All but Cel12 (Cel-12) and possibly Cel2 include a putative N-terminal signal peptide. Plant EGases have been identified that do not include a signal peptide and are presumably synthesized in the cytoplasm (Brummell *et al*., 1997*b*). The proteins in this subfamily of plant EGases include a membrane-spanning domain that putatively targets the protein to the plasma membrane where they may function in cell wall synthesis (Brummell *et al*., 1997*b*). Cel12 includes a putative membrane-spanning domain and has high sequence similarity with this subfamily of membrane associated EGases. Cel2, however, does not have a clearly defined membrane-spanning domain that could target the protein to the plasma membrane and does not have other characteristics common to this subfamily of EGases (Libertini *et al*., 2004). However, if the criteria for identifying a signal peptide are relaxed, an acceptable 31 amino acid signal peptide can be defined at the N-terminus of the deduced peptide sequence for Cel2. Nevertheless, it is possible that Cel2 is not synthesized on the endoplasmic reticulum and therefore its secretion into the cell wall is uncertain.

Gene expression in SCN-colonized roots

In addition to cell wall-modifying proteins, consensus sequences were obtained as described above for the soybean and SCN translation elongation factors (subunits 1 beta) and gene-specific primers prepared for each (see Supplementary Tables S1, S2 at *JXB* online). The soybean elongation factor, a constitutively expressed gene, was used in the RT-PCR experiments to normalize the RNA content in each sample. The SCN elongation factor was used to demonstrate that none of the non-inoculated root tissue was contaminated with nematode RNA.

The log base 2 (log2) ratios for all of the real-time PCR results are displayed as a heat-map in Fig. 1. This view of

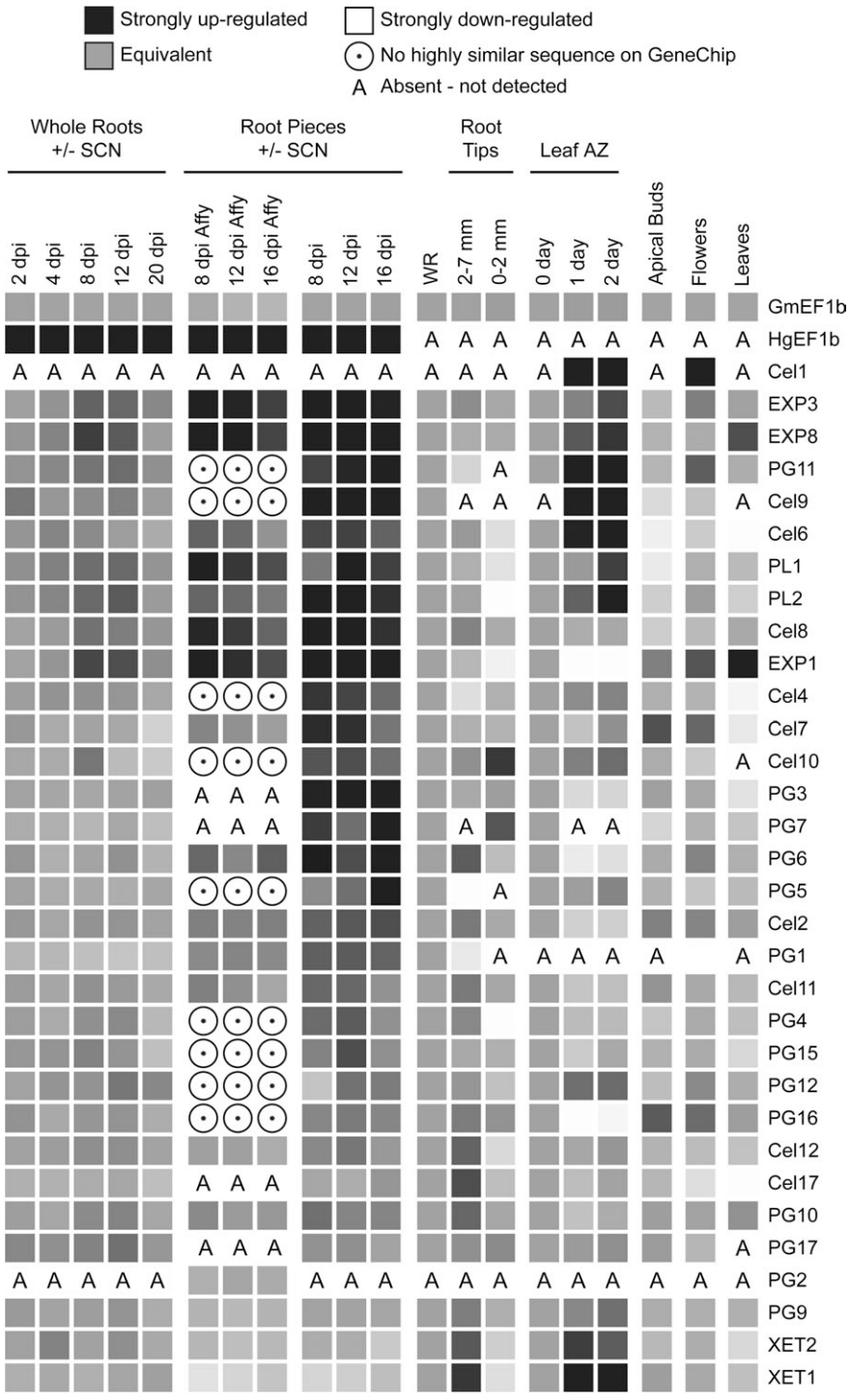


Fig. 1. A heat-map display of log2 ratios for the expression results of real-time RT-PCR and Affymetrix GeneChip hybridizations. A black box indicates a strong up-regulation, a neutral grey box equivalent expression, a white box a strong down-regulation, and shades of grey in-between indicate intermediate levels of regulation. Expression profiles for each gene were grouped by hand to cluster genes with similar expression profiles. RNA was isolated from whole roots and trimmed root pieces of SCN-colonized roots or non-inoculated roots at the indicated dpi, non-inoculated whole roots and root tips dissected at 0–2 mm and 2–7 mm proximal to the root apex, leaf abscission zones (AZ) from explants exposed to ethylene (0, 1 or 2 d), 2 mm of shoot apical buds, flowers at all stages of development, and young expanding leaves. The columns labelled Affy are log2 ratios for the GeneChip hybridizations. Where RNA was extracted from SCN-inoculated and non-inoculated whole roots or root pieces, the ratio is for similar root samples at the same dpi. For leaf AZ the ratio is the relative concentration for the labelled sample compared with the relative concentration at 0 d. The ratio for apical buds, flowers, and leaves are the relative concentrations for the labelled tissue sample compared with that for non-inoculated whole roots. Root tip dissections are relative to the expression in whole roots.

the results produces a good visual display of the general extent of the up- or down-regulation of gene expression and clustering of the gene expression profiles (Chiang *et al*., 2001). In the heat-map display it can be seen that many of the cDNAs for cell wall-modifying proteins were slightly up-regulated in whole roots infected with SCN and the difference between SCN-inoculated and non-inoculated roots was further enhanced in root pieces, which contained a greater proportion of developing nematodes per gram of root tissue collected (Fig. 1). The differential increase in transcripts in the SCN-colonized root pieces compared with whole roots indicates that these transcripts are not associated with a systemic defence response or a generalized response to the stress of being uprooted at the beginning of each experiment (i.e. 0 dpi) but is a localized response to the SCN infection.

To provide additional information with regard to the magnitude of the differences and the standard error of the means, linear and bar graphs are included in Fig. 2 for a few of the transcripts that were significantly up-regulated in SCN-infected roots (Cel8, EXP1, EXP3, EXP8, PG11, PL1, and PL2) and two genes whose expression profiles contrasted with the others (Cel1 and XET1). Cel1 is included because it is not expressed in any of the root RNA collected and XET1 because, in contrast to the many up-regulated genes identified, XET1 is down-regulated in the SCN-infected roots (Fig. 1).

The RNA samples collected from the SCN-colonized root pieces and non-inoculated root pieces were hybridized to Affymetrix GeneChip Soybean Genome Arrays (Puthoff *et al*., 2007). The PIDs (target sequences) with high sequence identity (>90%) to the cell wall-modifying transcripts described above are listed in Table 1. The reason for including in Table 1 sequences of less than 100% identity was because many of the consensus sequences used as target sequences on the GeneChip have positions within the sequences where no consensus nucleotide could be assigned and were therefore marked with the letter n. In our alignments an 'n' is counted as a mismatch. Many of the transcripts identified in soybean roots using the degenerate primers for EGases and PGs did not match any target sequence included on the GeneChip (i.e. Cel4, Cel9, Cel10, PG4, PG5, PG11, PG12, PG15, PG16; indicated in Fig. 1 as a circle with a dot inside).

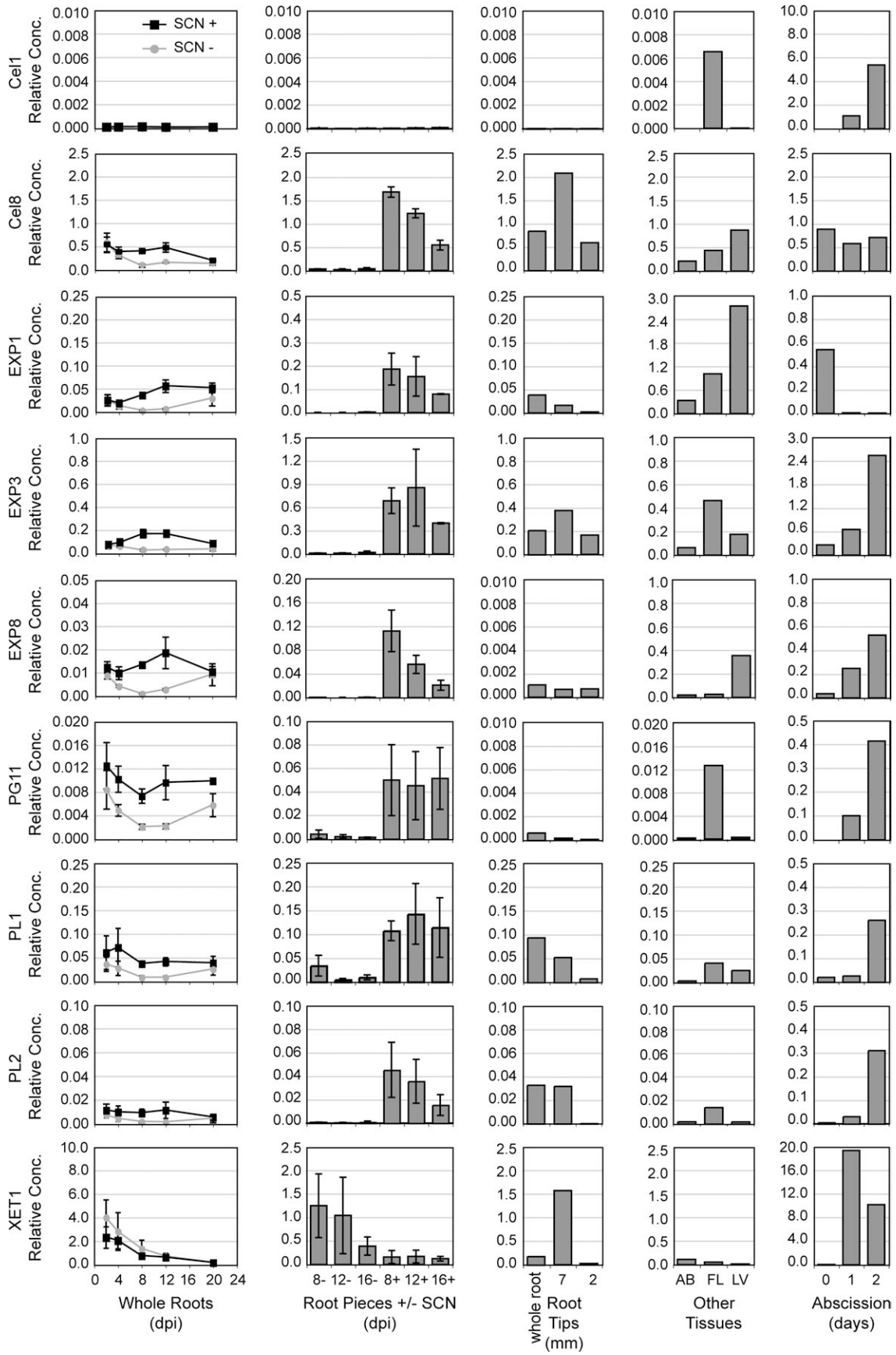
The GeneChip hybridizations produced very similar results as the RT-PCR results (Fig. 1). Moreover, the down-regulation of XET1 was quite notable for the GeneChip hybridizations because only eight PIDs out of 37 593 soybean PIDs on the GeneChip were significantly ($Q < 0.05$) down-regulated more than 8-fold ($\log2 \leqslant 3$) (Puthoff *et al*., 2007) and one of these had high sequence identity with XET1 (Table 1A). Included in Table 1B are the results for hybridization to target sequences with high sequence identity to the soybean and SCN elongation factors used for the design of PCR primers. The log2 ratios for PIDs similar to GmEF1b are close to 0, which indicates that there was very little difference in the expression of these genes between the SCN-colonized root pieces and the non-infected root pieces. However, the log2 ratio for the HgEF1b PID is at the extreme upper limit of all of the log2 ratios obtained for the GeneChip hybridizations because, although a signal is recorded for this PID in the non-inoculated root pieces, the hybridization signal is equal to or below background and considered absent in the RNA sample, i.e. $P > 0.05$.

RT-PCR expression results in root tips and other tissues

When the lateral roots were trimmed from the non-inoculated root pieces, the expression level of several cell wall-modifying genes dropped to nearly undetectable levels (Fig. 2). This suggested that, in whole roots, these RNAs might be concentrated in the lateral root tips and that by cutting out pieces and removing the lateral roots the primary source of these mRNAs in non-inoculated roots was removed. To examine the expression profile for these genes in roots further, whole roots and lateral root tips from the same batch of 18-d-old greenhouse-grown plants were collected. The root tips were then dissected into serial sections of 0–2 mm, and 2–7 mm proximal to the apex of root tip (Fig. 3). Important to understanding the accumulation of RNAs and their patterns of expression is that the first 2 mm of the root tip, which includes the root meristem, yielded approximately 10-fold more RNA per gram than the 2–7 mm fraction and 50-fold more RNA per gram than that of whole roots. The pattern of expression adjusted to the concentration of a constitutive control mRNA (GmEF1b) in these root tip fractions is quite complex (Figs 1, 2) and will be discussed in greater detail below in relation to developmental processes and previous results.

In order to identify other tissues and developmental processes that share a similar set of up-regulated genes, RNA was isolated from petiole abscission zones exposed to ethylene, apical buds, flowers of all developmental stages, and young expanding leaves. Several of the cell wall-modifying genes that were up-regulated in the SCN-infected root pieces were also strongly up-regulated in

Fig. 2. Graphical presentation of the linear gene expression profiles of normalized real-time RT-PCR results. Results for roots inoculated with SCN are indicated with black boxes and solid black lines and results for non-inoculated roots are indicated with grey circles and solid grey lines. Note that the scale for the relative concentration of the real-time RT-PCR products was kept as constant as possible; nevertheless, the scale changes for some in order to illustrate differences in gene expression better when they were much greater or less than the others. The means and standard error bars for whole roots are for three separate experiments and those for root pieces are for two experiments. Means without error bars are for pooled samples. Apical buds (AB), flowers (FL), and leaves (LV).


SCN +
SCN -
Cel1 Relative Conc.
Cel8 Relative Conc.
EXP1 Relative Conc.
EXP3 Relative Conc.
EXP8 Relative Conc.
PG11 Relative Conc.
PL1 Relative Conc.
PL2 Relative Conc.
XET1 Relative Conc.
0 4 8 12 16 20 24
Whole Roots (dpi)
8- 12- 16- 8+ 12+ 16+
Root Pieces +/- SCN (dpi)
whole root 7 2
Root Tips (mm)
AB FL LV
Other Tissues
0 1 2
Abscission (days)

abscission zones induced to abscise with ethylene (Fig. 1). Curiously though, the XET1 and XET2 transcripts that were down-regulated in SCN-infected roots were quite strongly up-regulated early in abscission zones (Figs 1, 2).

Discussion

Soybean gene transcripts for proteins that modify the plant cell wall were identified and sequence-specific PCR primers prepared. RT-PCR indicated that many of these genes were strongly up-regulated during SCN colonization of soybean roots. GeneChip hybridization experiments further supported this observation (Puthoff *et al*., 2007). Less than 1% of the GeneChip target sequences (gene transcripts) that were expressed in roots were up-regulated more than 8-fold in the SCN-colonized root pieces (Puthoff *et al*., 2007). However, more than 10% of the transcripts for cell wall proteins increase more than 8-fold in the SCN-colonized root pieces. These results highlight the importance of cell wall modification to the colonization of soybean roots by SCN. Recent reports for cyst nematode-infected *Arabidopsis* (Wieczorek *et al*., 2006) and SCN-infected soybean (Ithal *et al*., 2007*a*, *b*) support this conclusion.

Several of the root cDNAs encoding cell wall-modifying proteins that were identified using the degenerate PCR primers did not have high identity (>90%) with any consensus or target sequences used to design probe sets (PIDs) for the soybean GeneChip (Fig. 1). A Blast search of the GenBank soybean EST database (July, 2006) revealed that these same cDNAs either had no similar sequence, only one related EST or a very few sequences that aligned poorly. Of particular interest is PG11, which is currently a novel sequence for soybean, and was strongly up-regulated in SCN-colonized roots, flowers, and ethylene-induced abscission zones (Fig. 2).

At the start of this project it was hypothesized that SCN might induce cell wall-associated genes that were not normally expressed in the root and therefore would be unique to SCN-colonized roots. Although some of the genes examined were not abundantly expressed in non-inoculated roots, no gene was identified that was not expressed somewhere in the root system. These results suggested to us that SCN takes advantage of regulatory mechanisms and gene expression that already exist in the root system. It is the special combination of nematode and host regulatory factors in a limited space that creates the conditions necessary to establish the nematode feeding structure.

However, recently Wieczorek *et al*. (2006) surveyed 29 *Arabidopsis* EXPs for expression in roots colonized with the cyst nematode *Heterodera schachtii*. They identified 10 EXPs expressed in a syncytium cDNA library and used promoter GUS constructs to examine the expression patterns of seven of the EXPs in cyst nematode-infected *Arabidopsis* roots. Three of the EXPs, AtEXPA3, AtEXPA10, and AtEXPA16, displayed GUS staining that was restricted solely to the syncytium and not in any tissue above or below the syncytium. The construct for AtEXPA10 produced a faint GUS stain near the root tip. They concluded that AtEXP3 and AtEXP16 were specifically expressed in the syncytium and nowhere else in the root. GUS stain for the remaining four EXPs was observed to be strong in the syncytium but was also clearly visible in nearby vascular tissue, at the base of lateral roots, or root tips.

The EXPs are an unusual class of cell wall proteins because they aid in the extension of plant cell walls but have no known enzymatic activity (Sampedro and Cosgrove, 2005). The expression of three soybean EXPs (EXP1, EXP3, and EXP8) were examined by RT-PCR and all were strongly up-regulated by SCN (Table 1; Figs 1, 2). All three also produced a PCR product with the same melting temperature in non-inoculated whole roots as that observed in the SCN-inoculated roots. Although it is still possible that SCN initiates gene expression not normally found in the roots of soybean, the results for these three EXPs supports our conclusion that SCN utilizes regulatory mechanisms for the formation of the feeding structure that already exists in the soybean root.

Many of the developmental processes associated with roots occur within the first few millimetres of the root tip (Birnbaum *et al*., 2003). It was hypothesized that examination of gene expression in the root tip might provide clues to the host genetic mechanisms co-opted by the nematode to form its feeding structure. At the apex of the root tip is the root cap that protects the meristem and undergoes extensive cell wall modifications that results in the shedding of cells to produce a mucilaginous material that may aid in penetration of the root through the soil (del Campillo *et al*., 2004). Directly underneath the root cap is the root meristem, which involves synthesis of the cell plate and primary cell wall (Molhoj *et al*., 2002). Both of these tissues reside within the first 1 mm of the root tip (Fig. 3). Root hair development begins between 1–2 mm from the root tip but is most notable in the 2–7 mm section and continues further into the root becoming quite profuse at 12 mm (Fig. 3). Moreover, root hair density appeared to increase for several centimetres proximal to the root tip (data not shown). Cell elongation and vascular differentiation begins within the first 2 mm and extends into the next 5 mm (2–7 mm) of the root tip.

The normalized expression levels for most of the cell wall genes examined were highest in the first 7 mm of the root tip compared to their concentration in the rest of the root. For example, the relative expression of Cel4, Cel10, and PG7 were most highly expressed in the 0–2 mm portion of the root tip (Fig. 1), which includes the root cap, meristem, and initial phases of vascular differentiation. However, the greatest number of the cell wall associated genes examined, for example, Cel2, Cel8, Cel11, Cel12,

Table 1. *Gene expression results for Affymetrix soybean GeneChip PIDs associated with transcripts for the cell wall-modifying enzymes described in the text (A) and target sequences for soybean and SCN elongation factors, EF1B (B)*

Log2 ratios are for SCN-colonized root pieces relative to non-inoculated root pieces. If the hybridization signal strength was less than a statistical threshold ($P \geqslant 0.05$) for either biological replication, the log2 ratio column is marked with an A for absent. The *Arabidopsis* Gene Annotation indicates the putative function of the *Arabidopsis* gene with the greatest similarity to the soybean GeneChip target sequence. The cDNA Sequence Identity indicates sequence name, range of sequence identity, and per cent sequence identity to the Affymetrix target sequence (PID). *Q*-values are the False Discovery Rates for the SCN treatment effect (Puthoff *et al*., 2007). A *Q*-value $\leqslant 0.05$ was considered to be significantly different. ND, not determined.

(A) PIDs for sequences most similar to those used for primer preparation (Table S2).

PID	Log2 ratios			*Q*-value	*Arabidopsis* gene annotation	cDNA sequence identity
	8 dpi	12 dpi	16 dpi			
GmaAffx.93176.1.S1_at	5.7	4.7	4.4	0.05	Expansin (exp3)	GmEXP3, 99–597 (96%)
GmaAffx.93176.1.S1_s_at	7.2	5.1	4.6	0.00	Expansin (exp3)	GmEXP3, 99–597 (96%)
Gma.7784.1.A1_at	6.5	4.9	4.1	0.01	Expansin (exp1)	GMEXP1, 860–1314 (98%)
GmaAffx.90009.1.S1_s_at	5.4	5.3	4.2	0.00	Expansin (exp8)	GmEXP8, 320–778 (98%)
GmaAffx.85557.1.S1_at	5.4	4.1	3.3	0.01	Expansin (exp1)	GmEXP1, 50–507 (98%)
Gma.1326.3.S1_at	5.3	4.3	4.9	0.03	Pectate lyase	GmPL1, 258–709 (98%)
Gma.5785.2.S1_at	5.0	4.6	3.5	0.00	Cellulase	GmCel8, 128–406 (95%)
Gma.7006.1.S1_at	4.8	5.6	3.4	0.00	Expansin (exp4)	GmEXP3, 723–1218 (99%)
Gma.5785.1.S1_at	4.7	4.0	2.6	0.00	Cellulase	GmCel8, 2328–2476 (98%)
GmaAffx.91087.1.S1_s_at	4.6	4.4	3.0	0.00	Cellulase	GmCel8, 1818–2260 (94%)
Gma.1326.1.S1_at	4.6	5.0	3.0	0.00	Pectate lyase	GmPL1, 1331–1512 (99%)
Gma.1326.1.S1_a_at	4.2	3.4	2.3	0.03	Pectate lyase	GmPL1, 1331–1512 (99%)
GmaAffx.91087.1.S1_at	4.1	3.9	2.8	0.01	Cellulase	GmCel8, 1818–2260 (94%)
GmaAffx.78077.1.S1_at	3.8	2.6	2.0	0.00	No similar sequence	GmCel7, 1737–1983 (99%)
Gma.13475.1.S1_at	3.1	1.4	4.1	0.13	Polygalacturonase	GmPG6, 757–1050 (96%)
GmaAffx.20156.1.S1_s_at	3.0	2.0	3.1	0.08	No similar sequence	GmPG6, 1126–1250 (98%)
GmaAffx.62345.1.S1_at	2.8	2.6	1.2	0.04	Hydrolase family 9	GmCel6, 379–604 (95%)
GmaAffx.37672.1.A1_at	2.7	2.7	2.3	0.00	No similar sequence	GmPL2, 1264–1537 (98%)
Gma.2151.1.S1_at	1.8	1.3	0.3	0.16	Hydrolase family 9	GmCel11, 824–1293 (98%)
GmaAffx.79075.1.S1_at	1.7	1.8	2.0	0.00	Hydrolase family 9	GmCel2, 1136–1597 (99%)
Gma.41.1.S1_at	A	A	A	ND	Hydrolase family 9	GmCel1, 1134–1678 (99%)
GmaAffx.61171.1.S1_at	A	A	A	ND	Polygalacturonase	GmPG7, 796–1168 (97%)
Gma.16122.1.S1_at	1.6	1.1	0.8	0.00	Hydrolase family 9	GmPG7, 1327–1771 (91%)
GmaAffx.86997.1.A1_at	A	A	A	ND	No similar sequence	GmCel17, 1573–1692 (98%)
Gma.8494.1.S1_at	1.5	1.4	1.3	0.17	Polygalacturonase	GmPG1, 1061–1641 (100)
GmaAffx.25010.1.S1_at	A	A	A	ND	Polygalacturonase	GmPG3, 1408–1647 (92%)
Gma.7587.1.S1_at	1.3	0.7	1.0	0.05	Polygalacturonase	GmPG10, 862–1297 (98%)
Gma.12293.1.S1_at	A	A	A	ND	Polygalacturonase	GmPG17, 853–1121 (92%)
GmaAffx.36659.1.S1_s_at	1.2	1.8	1.9	0.17	Polygalacturonase	GmPG1, 547–765 (94%)
Gma.13475.2.S1_at	0.9	1.1	1.7	0.01	Polygalacturonase	GmPG6, 564–623 (98%)
GmaAffx.90341.1.S1_at	0.6	0.6	0.1	0.24	KORRIGAN/cellulase	GmCel12, 1030–1481 (99%)
Gma.16587.2.S1_a_at	0.5	0.5	–0.1	0.34	KORRIGAN/cellulase	GmCel12, 1622–1742 (99%)
GmaAffx.90341.1.S1_s_at	0.4	0.5	0.0	0.30	KORRIGAN/cellulase	GmCel12, 990–1452 (99%)
GmaAffx.91310.1.S1_at	0.4	0.7	0.3	0.10	KORRIGAN/cellulase	GmCel12, 1030–1491 (99%)
Gma.16587.1.S1_x_at	0.4	0.1	–0.4	0.48	KORRIGAN/cellulase	GmCel12, 1704–1993 (94%)
GmaAffx.91310.1.S1_s_at	0.3	0.9	0.3	0.14	KORRIGAN/cellulase	GmCel12, 411–1452 (99%)
Gma.16638.1.A1_at	A	A	A	ND	Polygalacturonase	GmPG17, 839–1121 (95%)
GmaAffx.86463.1.S1_at	0.0	0.0	0.1	0.27	KORRIGAN/cellulase	GmCel5, 875-1223 (99%)
GmaAffx.39763.1.S1_at	0.0	0.2	0.4	0.34	KORRIGAN/cellulase	GmCel5, 1–336 (94%)
Gma.8493.1.S1_at	–0.2	0.7	0.2	0.47	Polygalacturonase	GmPG2, 1087–1647 (100%)
GmaAffx.87008.1.S1_at	–0.6	–0.8	–0.4	0.05	Polygalacturonase	GmPG9, 1267–1542 (93%)
GmaAffx.36659.1.S1_at	–0.7	–0.3	0.1	0.22	Polygalacturonase	GmPG2, 583–819 (95%)
Gma.4051.1.S1_at	–0.9	–0.7	–0.9	0.15	Endo-xylo *trans*	GmXET2, 421–743 (95%)
Gma.2091.1.S1_at	–3.1	–2.3	–1.4	0.08	Endo-xylo *trans* (XTR6)	GmXET1, 688–1152 (97%)
GmaAffx.93483.1.S1_s_at	–3.4	–2.7	–1.3	0.15	Endo-xylo *trans* (XTR3)	GmXET1, 31–291 (100%)

(B) PIDs for translation elongation factors most similar to those used for data in Fig. 1.

Probe ID	Log2 ratios			Q-value	*Arabidopsis* gene annotation	cDNA sequence identity
	8 dpi	12 dpi	16 dpi			
GmaAffx.1301.31.S1_s_at	0.0	–0.2	–0.2	0.14	Soy elongation factor 1B	GmEF1b, 711–834 (100%)
GmaAffx.90455.1.A1_s_at	0.1	–0.4	–0.2	0.20	Soy elongation factor 1B	GmEF1b, 268–609 (100%)
GmaAffx.90455.1.S1_at	–0.4	–1.0	A	0.15	Soy elongation factor 1B	GmEF1b, 266–753 (99%)
HgAffx.24196.1.S1_at	8.5	9.4	8.6	ND	SCN elongation factor 1B	HgEF1b, 37–473 (99%)

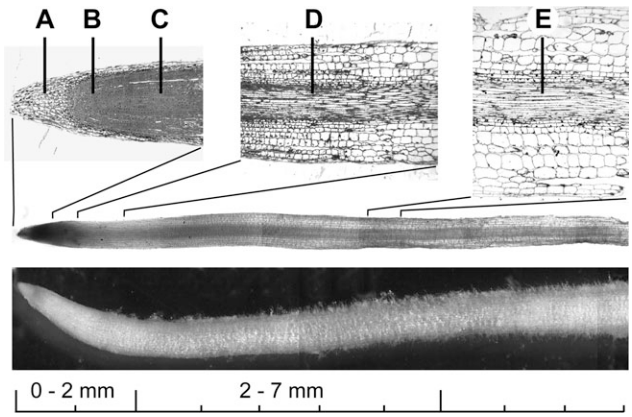


Fig. 3. Anatomy of a soybean lateral root tip. Intact root (bottom); 50 μm longitudinal section (middle); 0.5–1.0 μm plastic embedded sections (top). (A) Root cap, (B) meristem, (C) differentiating vascular system, (D) maturing vascular system, (E) nearly mature vascular system.

Cel17, EXP3, PG6, PG10, PG13, XET1, and XET2, were most highly expressed in the 2–7 mm portion of the root tip (Fig. 1), which includes the cell elongation zone, maturation of vascular tissue, and root hair elongation (Fig. 3). Cel12, a membrane-anchored EGase, which are proposed to be involved in cellulose biosynthesis (Brummell *et al*., 1997*a*, *b*; Molhoj *et al*., 2002), is most abundantly expressed in the 2–7 mm section of the root tip and also slightly elevated in SCN-colonized root pieces (Figs 1, 2). A membrane anchored EGase (*NtCel2*) was previously identified as being up-regulated in tobacco roots infected with RKN and TCN (Goellner *et al*., 2001).

In addition to *NtCel2*, Goellner *et al*. (2001) identified another cellulase that was up-regulated at the nematode infection site in tobacco roots that is orthologous to Cel8. Cel8 was also highly expressed in the 2–7 mm section of the soybean root tip and also strongly expressed in SCN-colonized root pieces (Fig. 2). Cel8, unlike the other EGases identified, includes a putative cellulose binding domain at its carboxy terminus (Trainotti *et al*., 1999). It has been proposed that a cellulose binding domain might enhance hydrolase activity against the xyloglucans that coat the cellulose microfibrils and cause a severe weakening of the cell wall by letting loose the cellulose microfibrils (Trainotti *et al*., 1999). However, a confirmed role for the cellulose binding domain in plants remains to be elucidated.

A very few genes, PG1, PG11, PL1, and PL2, appeared to have the highest relative expression in the whole root RNA and not in either of the two root tip fractions (Fig. 1). What processes occur in the whole root proximal to 7 mm are more difficult to assess. This region of the root includes radial expansion of the root, further maturation of vascular cells, and root hair development and growth. In addition, an occasional branch root was observed to emerge from the lateral root between 15–20 mm proximal to the root apex. The collection of root pieces was purposely limited at 7 mm so as not to collect branch roots that would essentially duplicate the 0–2 mm root tip collection. One process associated with the early growth of branch roots that does not occur in the root tip is the separation of the cortical cells during the eruption of branch roots through the root cortex. Mergemann and Sauter (2000) demonstrated that, in rice, the emergence of the lateral root was preceded by cell death external to the tip of the root primordia. Cell death proceeded until the epidermis split. In addition, they demonstrated that the cell death and splitting was most closely linked to ethylene and the ethylene-signalling pathway. A correlation between ethylene and the splitting of hypocotyls during the emergence of adventitious roots in soybean has also been reported (Kemmerer and Tucker, 1994). It is possible that cell wall modification precedes cell death and that a different set of gene expression is linked to the splitting of the cortex than that observed in the freely growing root tip.

Although the primary focus of this research project was on gene expression associated with SCN colonization of soybean roots, it is noteworthy that many of the same genes up-regulated by SCN were also up-regulated during ethylene-induced petiole abscission. PG11, PL1, and PL2 have expression patterns in abscission zones and flowers very much like Cel1 (Fig. 2), a previously characterized abscission-specific EGase (Kemmerer and Tucker, 1994; Koehler *et al*., 1996). The flowers used in these experiments included many stages of development that very likely included abscising pedicels, petals, and other abscising floral organs. PG11, PL1, and PL2 are notable in this context because, as mentioned above, their expression profiles indicated a higher relative expression in whole roots than the first 7 mm of the root tip. These shared expression profiles may indicate a shared mechanism of regulation that might be of interest in deciphering regulatory elements in their gene promoters.

With regard to abscission, it is interesting that XET1 expression was at its highest level in abscission zones after only one day of ethylene treatment and then declined by the second day (Fig. 2). These expression profiles are in contrast to the expression profiles for Cel1, PG11, and PL2, which all began to rise after 1 d of ethylene exposure and increase considerably more after 2 d of ethylene exposure (Fig. 2). The early expression of XET1 in abscission zones may indicate a special role for this enzyme in ethylene evoked cell wall modifications. Curiously though, XET1 declined in SCN-infected root pieces (Fig. 1; Table 1), which is also an ethylene-dependent process (Wubben *et al*., 2001). However, although XET1 transcript levels significantly declined in our experiments, other XTH genes have been demonstrated to increase in syncytia (Ithal *et al*., 2007*b*). The different XTH gene products may play very specialized roles in cell wall modifications and the down-regulation of one may be necessary for the optimal functioning of another.

An association has been demonstrated between SCN colonization of soybean roots and the change in gene expression for several soybean transcripts for cell wall-modifying proteins. The EXPs are noteworthy because it was demonstrated that the orthologous *Arabidopsis* transcripts increase specifically in the developing syncytium of *Arabidopsis* roots colonized by *Heterodera schachtii* (Wieczorek *et al*., 2006). Moreover, laser capture microdissection of syncytia from SCN-infected soybean roots included several EXPs (Ithal *et al*., 2007*b*). It is expected that several of the soybean transcripts characterized here will also increase specifically in the syncytium where cell wall degradation is extensive; however, the precise location of the increase in the soybean transcripts remains to be established. Nevertheless, a specific accumulation of transcript outside the syncytium would also be interesting. Secretion of proteins from the nematode into a host cell near the vascular bundle is proposed to initiate the process of syncytium development (Bird, 2004; Davis *et al*., 2004). Formation of the syncytium might well trigger responses in surrounding cells that could evoke changes in gene expression associated with cell wall modification. No matter the origin of the signal, changes in gene expression inside and outside the syncytium may be vital to the development of a functional nematode feeding structure.

Supplementary data

Supplementary data can be found at *JXB* online.

Table S1. Degenerate primers used to amplify cDNA fragments for cell wall-modifying proteins and gene-specific primers used in real-time RT-PCR. Nested degenerate primers are labelled with an N.

Table S2. FASTA file of all the sequences used for gene-specific primers and alignments with Affymetrix target sequences.

Acknowledgements

We thank Anil Neelam for suggestions and comments relating to the preparation of this research article. We also wish to thank Brandon Le, Anhthu Bui, and Bob Goldberg for graciously giving us their annotation file for the soybean GeneChip sequences and also Min Li and Steve Clough for a similar annotation file.

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